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# The *Escherichia coli* TatABC System and a *Bacillus subtilis* TatAC-type System Recognise Three Distinct Targeting Determinants in Twin-arginine Signal Peptides

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The Tat system transports folded proteins across bacterial and thylakoid membranes. In Gram-negative organisms, it is encoded by *tatABC* genes and the system recognizes substrates bearing signal peptides with a conserved twin-arginine motif. Most Gram-positive organisms lack a *tatB* gene, indicating major differences in organisation and/or mechanism. Here, we have characterized the essential targeting determinants that are recognized by a *Bacillus subtilis* TatAC-type system, TatAdCd. Substitution by lysine of either of the twin-arginine residues in the TorA signal peptide can be tolerated, but the presence of twin-lysine residues blocks export completely. We show that additional determinants can be as important as the twin-arginine motif. Replacement of the –1 serine by alanine, in either the TorA or DmsA signal peptide, almost blocks export by either the *B. subtilis* TatAdCd or *Escherichia coli* TatABC systems, firmly establishing the importance of this –1 residue in these signal peptides. Surprisingly, the +2 leucine in the DmsA signal peptide (sequence SRRGLV) appears to play an equally important role and substitution by alanine or phenylalanine blocks export by both the *B. subtilis* and *E. coli* systems. These data identify three distinct determinants, whose importance varies depending on the signal peptide in question. The data also show that the *B. subtilis* TatAdCd and *E. coli* TatABC systems recognize very similar determinants within their target peptides, and exhibit surprisingly similar responses to mutations within these determinants.

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## Introduction

The twin arginine translocation (Tat) pathway is involved in the transport of proteins across the chloroplast thylakoid membrane and the plasma membranes of a wide range of bacteria.<sup>1,2</sup> Operating in parallel with Sec-type protein transport systems, a notable feature of the Tat system is its ability to transport prefolded proteins; this form of translocat-

tion mechanism differs in fundamental respects from that of the Sec translocase, where substrates are threaded through the membrane in an unfolded state. In general, it appears that the Tat pathway is required for the transport of proteins that fold too rapidly or tightly to be transported in a Sec-dependent manner, and for substrates that bind complex redox cofactors before transport to the periplasm.

Tat-dependent signal peptides are surprisingly similar to Sec-type signal peptides and both comprise three distinct domains: an N-terminal positively charged domain (N domain), hydrophobic core domain (H domain) and more polar C-terminal domain (C domain) ending with the Ala-Xaa-Ala consensus motif that specifies cleavage by signal

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Abbreviations used: GFP, green fluorescent protein; YFP, yellow; WT, wild-type.

peptidase. However, Tat signal peptides contain a key RR-motif at the junction between the N and H domains, and mutagenesis experiments have shown this to be important for translocation. In chloroplasts, substitution of either arginine, even by lysine, results in a complete block in translocation by the thylakoidal Tat system.<sup>3,4</sup> Among bacterial Tat systems, the well-studied *Escherichia coli* Tat system has rather less stringent requirements; substitution of either arginine by lysine leads to a modest reduction in export rate, while substitution of both arginine residues blocks translocation.<sup>5</sup> However, an intact twin-arginine is required for efficient export of one Tat substrate in *Zymomonas mobilis*.<sup>6</sup>

Studies on the *E. coli* and plant Tat systems suggest that they operate in a generally similar manner. Both are minimally encoded by three genes, termed *tatABC* in *E. coli* and *tha4*, *hcf106* and *cpTatC* in chloroplasts.<sup>7–10</sup> Their gene products form two distinct complexes. The first is a TatABC complex containing multiple copies of these subunits, with the TatB and TatC subunits arranged in a tight heterodimeric unit.<sup>11</sup> The *E. coli* TatABC complex has a mass of about 370 kDa as judged by blue-native gels,<sup>12</sup> and *in vitro* translocation assays have shown that substrates bind initially to the TatBC subunits.<sup>13</sup> Thylakoid Tat substrates likewise bind to the homologous Hcf106 and cpTatC subunits.<sup>14</sup> Other crosslinking studies suggest strongly that the binding of substrate triggers recruitment of a separate, homo-oligomeric TatA complex (Tha4 complex in plants) to form the fully active translocon.<sup>15</sup> However, the actual translocation mechanism is poorly understood.

While the *E. coli* and plant Tat systems appear to be generally similar in structural and mechanistic respects, the vast majority of Gram-positive bacteria, with the exception of *Streptomyces* species,<sup>16</sup> contain Tat systems that lack a *tatB* gene and hence contain only the TatAC subunits.<sup>17</sup> The example under study here is *Bacillus subtilis*, a non-pathogenic, Gram-positive soil bacterium that secretes high levels of extracellular proteins. In contrast to *E. coli*, *B. subtilis* contains three variants of the *tatA* gene, *tatAd*, *tatAy* and *tatAc*. In addition, it contains two variants of *tatC*, *tatCd* and *tatCy*, while it lacks *tatB*. As TatA and TatB proteins share a high degree of sequence homology, it seems likely that the TatA proteins in *B. subtilis* are bifunctional and are performing the function of both the TatA and TatB proteins of *E. coli*.<sup>18</sup>

TatAd and TatAy have been shown to form two separate translocases with TatCd and TatCy, respectively, which display different substrate specificity.<sup>19</sup> The secretion of PhoD, a protein with phosphodiesterase and alkaline phosphatase activity, is completely TatAdCd-dependent.<sup>20</sup> *phoD* is located upstream of *tatAd* and *tatCd*, and these three genes appear to be expressed only under phosphate-limiting conditions, with no other TatAdCd-dependent substrate identified to date. TatAyCy has been shown to form an active translocase for the translocation of other substrates although only one,

YwbN, has been identified.<sup>19,21</sup> The function of the third TatA component, TatAc, is unknown, although it is not required for translocation of either PhoD or YwbN.<sup>19</sup>

Little is currently known about the mechanism or substrate specificity of the *B. subtilis* Tat system or any other TatAC-type system. We have recently found that the TatAdCd translocase is active in an *E. coli* *tat* deletion strain and able to translocate at least some *E. coli* Tat substrates (J.P.B. *et al.*, unpublished results). Here, we have used simple and direct export assays to probe the Tat signal determinants that are important for recognition by the TatAdCd system. The data show that three distinct determinants have important roles in signal peptide recognition by this Gram-positive system and by the *E. coli* Tat system.

## Results

### Targeting determinants recognized by the *B. subtilis* TatAdCd system

A twin-arginine motif in the signal peptide plays a key role in the initial interaction between a substrate and the Tat translocon, but the absolute importance of this motif can vary. In chloroplasts, an intact RR-motif is all-important,<sup>3,4</sup> whereas the *E. coli* Tat system does tolerate KR and RK-motifs, and disruption of both arginine residues is required to effect a full block in translocation.<sup>5</sup> Gram-positive Tat systems have been subjected to this form of study and a comprehensive study on the *Streptomyces lividans* system showed that the twin-arginine motif is an important determinant, as expected.<sup>22</sup> Substitution of both arginine residues by lysine was shown to substantially inhibit, but not block completely, the export of one substrate studied, and other residues in the signal peptide were also substituted (see below).

The Tat systems referred to above all contain three components (TatABC, or homologs thereof) but most Gram-positive organisms lack the TatB component that is essential in these systems and, instead, contain a different, two-component class of Tat system. Much less is known about the importance of the RR-motif in these systems, partly because efficient export assays have proved difficult to establish in model Gram-positive organisms. *B. subtilis*, for example, is difficult to study because only a single substrate has been identified for each of the two Tat systems; furthermore, this organism contains high levels of protease activity. We showed recently that the *B. subtilis* *tatAdCd* operon is active when expressed in an *E. coli* *tat* null mutant (J.P.B. *et al.*, unpublished results) and this enables us to use established export assays to study the requirements and characteristics of this Gram-positive Tat system. Pop *et al.* showed that a PhoD-LacZ fusion was exported under similar circumstances but the protease-sensitivity in spheroplasts of both the mature-size and processed forms makes this an

awkward assay for export.<sup>29</sup> We subjected the key elements of two Tat signal peptides to site-specific mutagenesis in order to characterise the essential determinants. In the experiments described below, substrates were expressed using the arabinose-inducible pBAD24 plasmid (with induction for 3 h) and the TatAdCd system was expressed from the compatible pEXT22 plasmid without induction. In parallel, we analysed export of these mutant substrates by the *E. coli* TatABC system by expressing the *tatABC* operon using the same pEXT22 plasmid. All experiments were carried out in the *AtatABCDE* background strain. The effects on export are summarized in Table 1, which also summarizes previous mutagenesis studies on Tat signal peptides from both Gram-negative and Gram-positive organisms for ease of comparison. Figure 1(a) shows the sequences of the *E. coli* TorA and DmsA signal peptides that were used in this study.

### Translocation by the TatAdCd system displays an absolute requirement for a single arginine in the twin-arginine motif

A particularly effective Tat export assay involves the use of a fusion protein comprising the signal peptide of *E. coli* TorA linked to green fluorescent protein (GFP). This TorA-GFP protein is exported

efficiently by the *E. coli* Tat system and no periplasmic protein is detected in control assays when the substrate is expressed in an *E. coli* *tat* null mutant (J.P.B. *et al.*, unpublished results).<sup>23</sup> The protein is then found only in the cytoplasmic and membrane fractions.

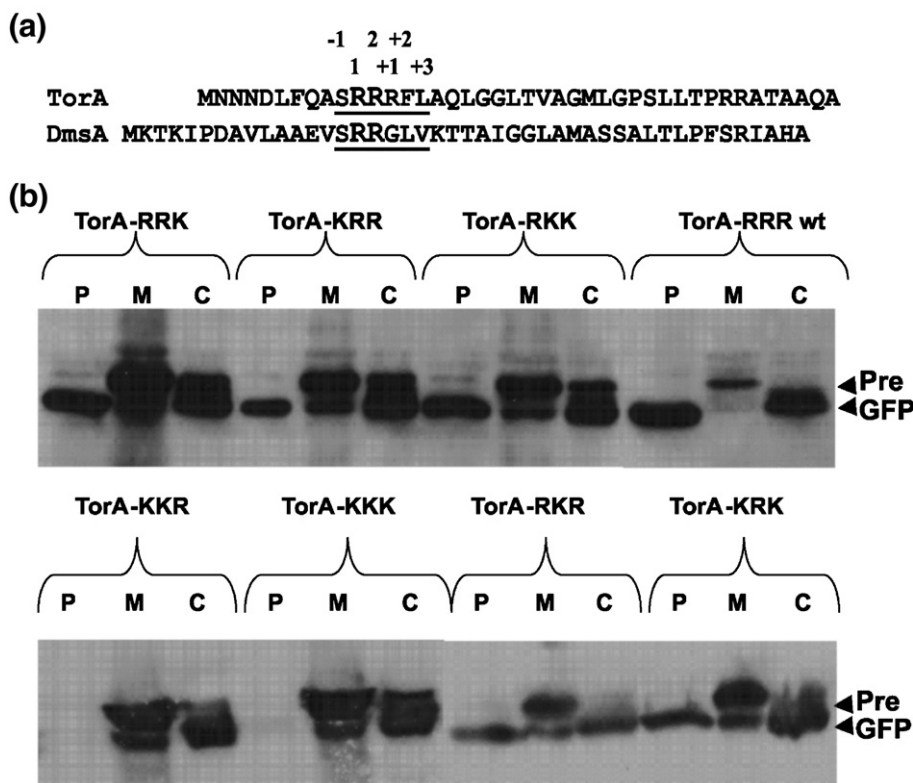
The importance of the twin-arginine was tested by substituting one or both arginine residues by lysine, and the results are shown in Figure 1(b). The sequence of the TorA signal peptide is shown in Figure 1(a), together with that of DmsA, which was also used in some experiments (see below). The sequence around the RR-motif is SRRRFL in TorA, and previous studies on the TorA signal peptide have shown that the first two arginine residues (underlined) form the RR-motif.<sup>24</sup> However, we also tested whether the positioning of the RR-motif is important for recognition by the *B. subtilis* TatAdCd system, by substituting the third arginine in some constructs (below). Expression of the wild-type (non-mutated) TorA-GFP construct is illustrated in the panel denoted TorA-RRR wt in Figure 1(b), and the data show that a significant proportion of mature-size GFP is found in the periplasm (lane P), indicating successful export. Some protein is found in the membrane fraction, often running as a smear on immunoblots, and this has been observed elsewhere.<sup>25</sup> Rapid induction of TorA-GFP often

**Table 1.** Summary of effects of mutations on Tat signal peptides

Organism/ substrate/motif	Sequence	Level of inhibition				Reference
		None	Mild-moderate	Severe	Complete	
<i>RR-motif</i>						
E.c. TorA-GFP	<u>SRRR</u> FL		<b>KR</b>			24, t.s.
E.c. SufI	<u>SRRQ</u> FI		<b>RK</b>		<b>KK</b>	24, t.s.
E.c. YacK	QRRDFL		RK		KK	5
E.c. TorA-MalE	<u>SRRR</u> FL			KK	KR	5
S.l. XlnC	SRRGFL		KR		KQ	31
Z.m. GFOR	<u>TRR</u> ALV			KK		22
				KR		31
					RK	31
<i>-1 residue</i>						
E.c. SufI	SRRQFI	S to A	S to C			5
E.c. TorA-GFP	<u>SRRR</u> FL			<b>S to A</b>		t.s.
E.c. DmsA-YFP	<u>SRRG</u> LV				<b>S to A</b>	t.s.
<i>+2 residue</i>						
E.c. SufI	SRRQ <u>F</u> I	F to L	F to A F to Y			5
E.c. TorA-GFP	SRRR <u>F</u> L	<b>F to A</b> <b>F to S</b>		<b>F to R</b>	<b>F to D</b>	5 t.s.
E.c. DmsA-YFP	SRRG <u>L</u> V			<b>L to F</b>	<b>L to A</b> <b>L to D</b>	t.s. t.s.
S.l. XlnC	SRRG <u>F</u> L		F to W	F to Y F to T F to H F to S F to K		t.s. 22 22 22 22

The Table shows the effects of mutating residues in Tat signal peptides, with emphasis on the RR-motif, the preceding (-1) residue and the subsequent +2 residue. The residue(s) in question are shown underlined in the six-residue sequences. Examples shown in bold illustrate the effects observed on the export of the mutant precursor by both *E. coli* TatABC and the *B. subtilis* TatAdCd systems studied here. Note that the levels of inhibition (mild-moderate, severe, etc.) are, in a sense, artificial because different forms of export assay were used in different studies. *E. coli*, *E. coli*; *Z. m.*, *Z. mobilis*; *S. l.*, *S. lividans*. t.s., this study.





**Figure 1.** Substitution of the twin-arginine motif by twin-lysine blocks TorA-GFP export by the *B. subtilis* TatAdCd system but single-lysine mutations are tolerated. (a) The sequence of the TorA signal peptide, together with that of DmsA, which is used in later experiments. (b) The TorA-GFP was expressed for 3 h in an *E. coli tat* null mutant strain that constitutively expresses *B. subtilis* TatAdCd from a separate plasmid. The TorA-RRR wt panel shows export of the non-mutated precursor (which contains an intact RR-motif plus subsequent +1 arginine residue). Other panels show the export of constructs containing mutated signal peptides with one or more of the arginine residues substituted by lysine as indicated. Membrane (M), cytoplasm (C) and periplasm (P) samples were prepared and analyzed by immunoblotting using anti-GFP antibodies. The mobilities of mature-size GFP and the precursor form (Pre) are indicated.

results in the accumulation of misfolded, inactive protein that binds to the membrane (lane M) and is slowly degraded.<sup>25</sup> We routinely observed smaller GFP polypeptides, often of near mature size, in the cytoplasm (C). This is due to proteolytic degradation of the precursor protein, which appears to be unavoidable with this substrate; this has been noted with TorA-GFP.<sup>25</sup> The extent of proteolysis differs widely between experiments for unknown reasons. The important point is that the fractionation procedure is effective.

The effects of mutating one of the RR-motif arginine residues are shown in the TorA-KRR and RKR panels. Neither of the single-lysine substitutions affects export efficiency significantly, and mature-size GFP is clearly present in the periplasm. However, substitution of both arginine residues (TorA-KKR panel) leads to a complete block in export. Clearly, substrate recognition and/or translocation is dependent on the presence of at least one arginine in this motif. We are unable to determine whether substitution of a single arginine causes a significant reduction in export kinetics, because the assay is insufficiently quantitative in terms of export rates. Nevertheless, the cells are fractionated after induction of TorA-GFP synthesis for only 3 h and

major effects on export efficiencies would be detected under these conditions.

The TorA signal peptide contains the sequence SRRRFL, and a combination of mutagenesis and bioinformatic studies has shown that the consensus sequence for bacterial Tat signals is SRRxFL, with the +2 Phe and +3 Leu highly prevalent, although not essential for translocation.<sup>5,26</sup> The TorA signal peptide thus conforms closely to a consensus Tat signal but in this case the +1 *x* residue following the twin-arginine motif is another arginine. This enabled us to test whether the twin-arginine motif has to be positioned precisely, relative to other determinants, in order to be recognised by this Gram-positive Tat system. Accordingly, we expressed a TorA-GFP mutant containing KKR in place of RRR, on the basis that the twin-arginine motif would be completely altered but the presence of the +1 arginine provides a KR motif. This KR motif would be shifted by one residue (towards the C terminus) compared to the KRR mutant, which is exported efficiently. An export assay with this KKR mutant is shown in Figure 1(b); the mutant is not exported to any detectable extent, demonstrating that the context of the RR-motif is critically important.

Previous studies have analyzed the transport of similar TorA-GFP mutants by the *E. coli* Tat system and the effects of the single/double lysine substitutions are similar to the effects shown here on export by TatAdCd.<sup>24</sup> We analyzed the export of the TatAdCd system with the above mutants in *E. coli* in order to compare the export requirements of this *B. subtilis* system with the well-characterized *E. coli* system under similar conditions and our data effectively confirmed the data reported by DeLisa *et al.*<sup>24</sup> The single-arginine mutants are exported with wild-type, or near wild-type efficiency, while the twin-lysine mutant is not exported to any detectable extent (data not shown).

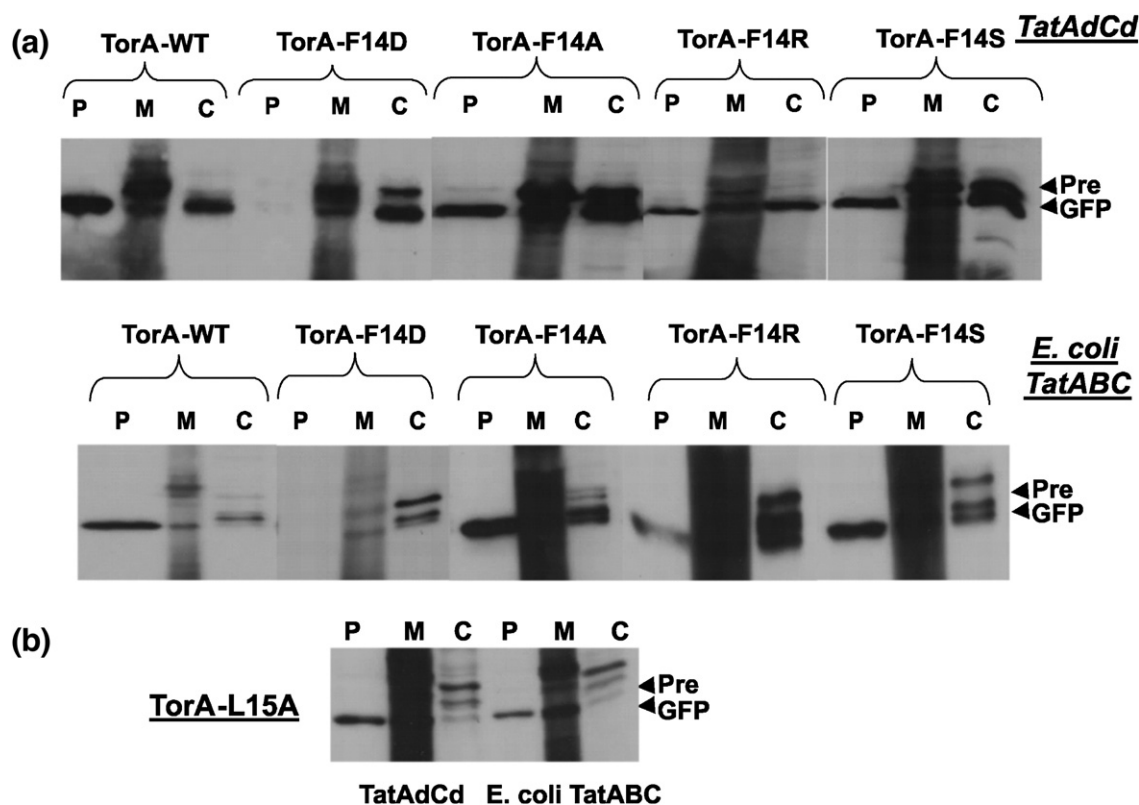
### The +2 phenylalanine in the TorA signal peptide is not required for targeting by the TatAdCd or TatABC systems

Phenylalanine is very commonly found in bacterial Tat signals at the +2 position,<sup>5,26</sup> and we tested whether this residue is important for transport by the TatAdCd system. This residue in TorA-GFP (Phe14) was substituted by a variety of residues and the data are shown in Figure 2. The results show that the F14A and F14S mutants are exported with efficiencies that resemble that obtained with the wild-type construct. However, an F14R mutant is exported with a much lower level of efficiency and substitution of Phe14 with aspartate (F14D) blocks

export completely. Clearly, phenylalanine is not a strict requirement at this position and removal of the hydrophobic side-chain in this signal peptide does not have a serious effect. However, an acidic side-chain at this position cannot be tolerated by the TatAdCd system.

Unlike the RR motif mutations, these TorA-GFP mutations have not been characterised in terms of their effects on export by the *E. coli* Tat system, so we studied their export using the same general expression system, with the substrates again expressed from the arabinose-inducible pBAD24 plasmid and the translocase (*E. coli* tatABC operon) expressed using the pEXT22 vector. The data (lower panel of Figure 2) show that the mutants have similar effects on export by the *E. coli* system; the F14A and F14S mutants are exported with efficiencies that closely resemble that of non-mutated TorA-GFP, the F14R mutant is exported with a much lower level of efficiency and the F14D mutant is not exported at all.

The +3 Leu in the TorA signal peptide was not studied in such detail, but we made one substitution in which it was replaced by Ala. The data (Figure 2(b)) show that the mutation has no detectable effect on export efficiency, and the protein is exported to a similar extent in cells expressing either TatAdCd or *E. coli* TatABC. A particularly hydrophobic residue is thus not a requirement at this position in the TorA signal peptide.



**Figure 2.** Effects of mutating the +2/+3 hydrophobic residues in the TorA signal peptide. The basic experiment was as shown in Figure 1, with mutated TorA-GFP constructs expressed in cells containing *B. subtilis* TatAdCd or *E. coli* TatABC as indicated. (a) The effects of mutating the +2 phenylalanine to arginine, aspartate, serine or alanine. (b) The effects of mutating the +3 leucine to alanine. Other symbols are as in Figure 1.

### Critical importance of the +2 Leu in the DmsA signal peptide

Most of our mutagenesis studies were carried out on the TorA-GFP substrate but we considered it important to analyze other signal peptides in case the important determinants vary between different signal peptides. In particular, the above data indicate that the twin-arginine is not the only key determinant in Tat signal peptides, since the presence of a KR motif is insufficient to support translocation when it is shifted by only a single residue. Previous studies on thylakoid signal peptides showed that translocation by the thylakoid Tat system is dependent on the presence of a highly hydrophobic residue at either the +2 or +3 positions after the RR-motif, and we initially focussed attention on this region.<sup>27</sup> We chose the DmsA signal peptide, in this case fused to YFP, because this signal peptide does not contain the FL consensus motif and instead has the sequence SRRGLV. The +2 Leu (Leu19) was substituted by several different residues and surprising results were obtained, as shown in Figure 3, with the upper panel containing data obtained with cells expressing TatAdCd.

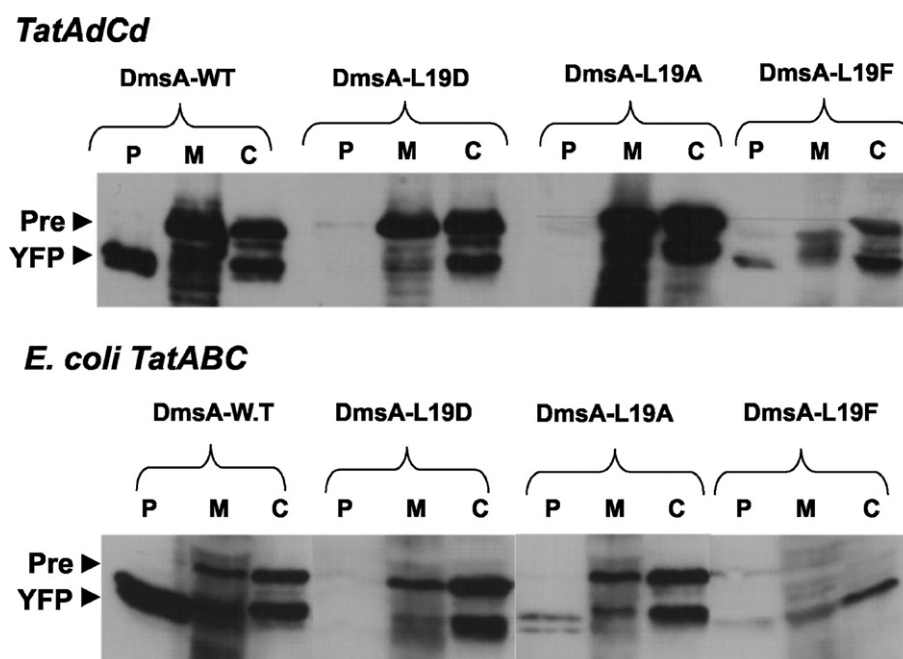
In control tests with the non-mutated DmsA-YFP construct (DmsA-WT panel) the protein is exported by TatAdCd and processed to the mature size, confirming that the TatAdCd system recognizes the DmsA signal peptide as well as the TorA signal. As with the TorA-GFP substrate, export is incomplete and some protein is present in the cytoplasm and membrane fractions. Substitution of the +2 leucine (Leu19) by aspartate (DmsA-L19D) causes a block in

export and no periplasmic protein is evident; this result is unsurprising, since a similar mutation at the corresponding position in TorA-GFP (F14D) had this effect. More interestingly, the L19A mutant is not exported to any detectable extent either, indicating clearly that this mutation profoundly affects the export-competence of this precursor protein. Also surprising is the finding that substitution by Phe (L19F panel) again causes a substantial inhibition of import; this mutant is exported very inefficiently indeed, despite the prevalence of Phe at this position in other Tat signal peptides. These data confirm the extreme importance of this region and suggest that different substitutions can have markedly differing effects depending on the composition of the surrounding sequence.

The same mutants were tested for export by *E. coli* TatABC (lower panel) and very similar data were obtained. The L19D is not exported at all, and the L19F mutant is exported very inefficiently. A minor difference is that very low-level export of L19A is observed (whereas no export of this mutant was detected with TatAdCd-expressing cells) but the export efficiency is very low indeed and this mutation almost blocks export. Clearly, this +2 residue plays a prominent role in recognition of the DmsA signal peptide by either the TatAdCd or TatABC systems.

### Importance of the -1 serine residue

As noted previously, the -1 residue directly preceding the RR motif is usually hydrophilic, and one that favours the capping at the N termini of



**Figure 3.** Substitutions of the +2 leucine in the DmsA signal peptide have a dramatic effect on export. A DmsA-YFP construct was expressed using the pBAD24 plasmid in *AtatABCDE* cells expressing TatAdCd (upper panel) or *E. coli* TatABC (lower panel). The Figure shows data from cells expressing the non-mutated precursor (DmsA-WT) and mutants containing aspartate, alanine or phenylalanine substitutions in place of Leu19.

helices, such as serine, threonine, aspartate or asparagine.<sup>5</sup> However, replacement of the -1 serine with alanine (which does not have this capping propensity) did not affect export kinetics of the SufI precursor in the study reported by Stanley *et al.*<sup>5</sup> Replacement with cysteine did cause a marked inhibition of export, suggesting that a more hydrophobic side-chain is detrimental at this position.<sup>5</sup>

We studied the importance of the -1 serine in both the TorA and DmsA signal peptides by substituting them with alanine, and the data are shown in Figure 4. Surprisingly, in view of the above SufI data, both mutations drastically inhibit export by the TatAdCd system, with only a very small proportion of the protein exported. The lower panel shows the two non-mutated substrates expressed in TatAdCd or TatABC-expressing cells, where a clear majority of the substrate is exported. The inhibition is even more pronounced when either of the -1 serine mutants is expressed in TatABC cells, to the extent that export is inhibited almost completely. These data provide firm evidence that the identity of the -1 side-chain can be very important for translocation, and suggest a vital role for a hydrophilic side-chain at this position within these signal peptides.

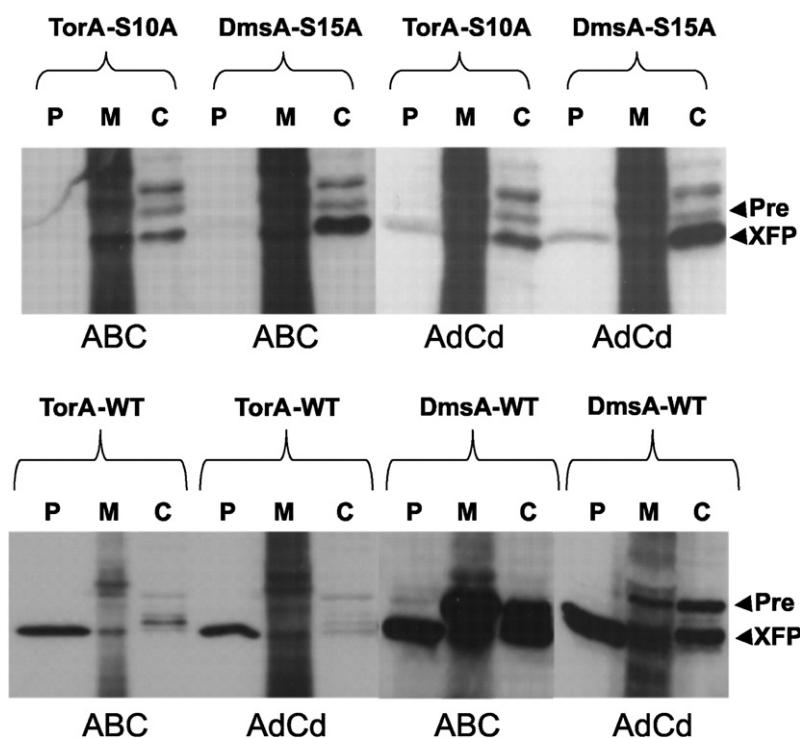
In view of the surprising findings with some of the mutated signal peptides, it was considered important to determine whether the observed export is mediated solely by the Tat pathway. We therefore analyzed all of the transport-competent mutants in a  $\Delta tatABCDE$  strain to address this question directly. The data (Figure 5) show that export is blocked in every case, confirming that any observed export can be attributed to the Tat pathway. This is perhaps not surprising, since there are indications that the Sec pathway has difficulty

transporting GFP,<sup>23</sup> but it is nevertheless useful to confirm this point experimentally.

### Bioimaging of Tat-dependent export

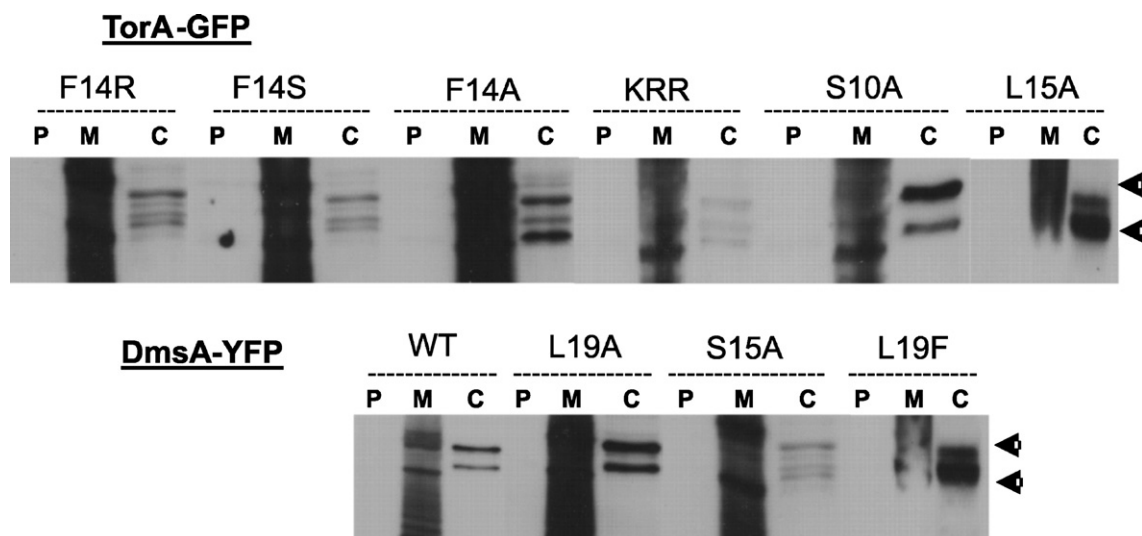
While fractionation and immunoblotting are effective means of assaying for Tat-dependent protein export, the use of GFP as a passenger protein provides an alternative means of studying the export process. Earlier, we used confocal microscopy to demonstrate Tat-mediated export of TorA-GFP to the periplasm,<sup>23</sup> and we used this approach with the mutants described above. Apart from the desirability of applying an alternative assay for this protein transport process, live cell imaging allows us to identify any anomalies that may arise during the expression of these GFP constructs. As an example, we found that expression of GFP-tagged Tat components sometimes leads to the appearance of punctate aggregates that are probably unphysiological.<sup>28</sup>

Figure 6 shows images of cells expressing TatAdCd together with TorA-GFP or DmsA-YFP. The non-mutated wild-type (WT) fusion proteins are visible as halos of fluorescence around the cells, consistent with their demonstrated location within the periplasm. Some cells appear brighter than others, with less well defined halos, and this is due to the arabinose-inducible pBAD promoter used in this study. We and others have found that individual cells become induced at different times, and to different extents, once arabinose is added to the medium. The remaining images show cells expressing TatAdCd together with the mutated precursors, and the data generally agree well with the fractionation data. For example, the above data show that all



**Figure 4.** The -1 serine residue is an important targeting determinant in both the DmsA and TorA signal peptides. The serine residue immediately preceding the twin-arginine motif in the DmsA signal peptide (Ser15) and that of TorA (Ser10) was substituted by alanine in the DmsA-YFP and TorA-GFP fusion proteins. Both were expressed in  $\Delta tatABCDE$  cells expressing TatAdCd or *E. coli* TatABC as indicated in the upper panel. Control assays were carried out using the wild-type constructs in the same strains (lower panel). Cells were fractionated into membrane (M), periplasm (P) and cytoplasm (C) samples as in previous Figures; the mobilities of the precursor forms of GFP/YFP are indicated (Pre) as are the mature proteins (XFP).





**Figure 5.** The mutated precursor proteins are exported exclusively by the Tat pathway. Translocation-competent TorA-GFP mutants (upper panel) together with DmsA-YFP and several of the mutated forms (indicated) were expressed in  $\Delta tatABCDE$  cells to test whether they can be translocated by the alternative Sec pathway. After expression of precursors for 3 h, cells were fractionated into membrane (M), cytoplasm (C) and periplasm (P) samples as in previous Figures. Arrowheads denote the expected mobility of the precursor and mature forms of the substrates.

of the DmsA-YFP mutations are severely affected in export (Figures 3 and 4) and the images likewise show no evidence of halos, with the YFP fluorescence distributed evenly in the cytoplasm.

Among the TorA-GFP mutants, TorA-L15A, TorA-F14A, and TorA-F14S exhibit clear halo patterns, whereas others (TorA-F14R, TorA-F14D, and TorA-S10A) show evenly distributed fluorescence. These data are consistent with the fractionation data that showed the former to be exported whereas the latter group are not.

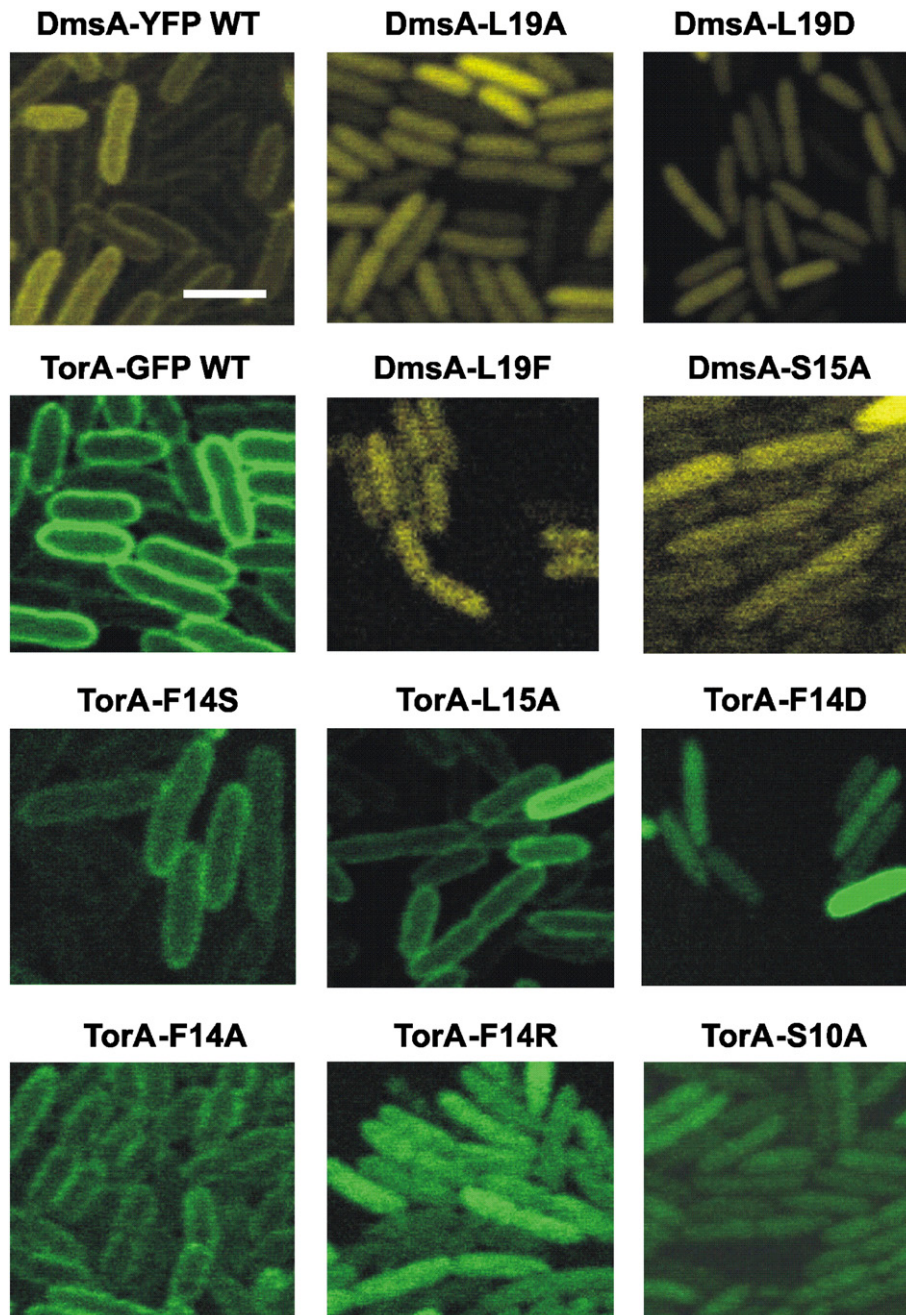
## Discussion

Previous studies have shown that the *E. coli* and plant systems recognise broadly similar signal peptides, but the two types of system do differ in terms of both signal peptide composition and the importance of the targeting determinants.<sup>3,4,5,22–24,26</sup> Bacterial signal peptides contain the consensus signal sequence SRRxFLK in which the twin-arginine is almost invariant, the +2 phenylalanine is very common (present in the majority of bacterial Tat signals) and the +4 lysine is also favoured. Plant Tat signals contain the twin-arginine motif but the FLK residues are rarely, if ever found. As discussed above, the two systems also display differing tolerances to mutations within the twin-arginine motif.

In this study we have sought to understand key basic aspects of Tat translocation signals recognized by a Tat system from the Gram-positive organism *Bacillus subtilis*. The absence of *tatB* genes from the majority of Gram-positive organisms indicates clearly that their Tat systems differ significantly from the *E. coli*/chloroplast model. In fact, other studies on *B. subtilis* TatAdCd have suggested a

radically different translocation model in which TatA, rather than TatBC, is the substrate-binding unit. Moreover, TatA is proposed to operate in the cytoplasm.<sup>29,30</sup> This model is fundamentally different from current models for the Tat operation in Gram-negative bacteria and plant thylakoids. Irrespective of the actual translocation mechanism, however, it is of interest to characterise the Tat signal peptide recognition process with a Gram-positive TatAC-type system for the first time.

In general, our data indicate that the TatAdCd system recognizes targeting determinants very similar to the previously-characterized *E. coli* system; each signal peptide mutation has broadly similar effects on export by both systems. Mutagenesis of the RR motif in TorA-GFP shows that this motif is important (as expected) but not essential for transport. The presence of twin-lysine leads to a complete block in export, but single-lysine mutations (the KRR and RKR mutants) are exported with reasonable efficiency. It is likely that these mutations slow the rate of export, but the effects are insufficiently dramatic to be evident over the 3 h of substrate induction used in this study. Overall, the results are reminiscent of those reported by Stanley *et al.*,<sup>5</sup> who observed similar effects on the *E. coli* TatABC system. However, the importance of the two arginine residues can vary, depending on the precursor and/or organism under study. Both arginine residues are required for the export of NADP-containing glucose-fructose oxidoreductase in *Zymomonas mobilis*;<sup>6</sup> in contrast, a twin-lysine motif still supported low-level, but detectable, export of xylanase in *S. lividans*.<sup>22</sup> It is also relevant to emphasise that in this discussion our definition of complete inhibition refers to an inability to detect export. It is likely that very low-level export is taking



**Figure 6.** Confocal microscopy of cells expressing TatAdCd together with DmsA-YFP or TorA-GFP mutants. Confocal images of  $\Delta tatABCDE$  cells expressing TatAdCd together with non-mutated forms of TorA-GFP or DmsA-YFP (denoted WT) or four versions containing the indicated mutations. The scale bar represents 1  $\mu\text{m}$ .

place with some of these “blocked” precursors, and indeed Kreuzenbeck *et al.*, using a highly sensitive growth assay, concluded that low-level export of a TorA-MalE construct with a twin-lysine motif must have taken place.<sup>31</sup>

Additional determinants clearly play a vital role in the functioning of a Tat signal peptide, with both the TatAdCd and *E. coli* TatABC systems. First, we observe a block in export when a KR motif is present but shifted towards the C terminus by a single residue (the KKR mutant shown in Figure 2). In one sense, a similar result was noted by Buchanan

*et al.*,<sup>32</sup> who found that the full TorA protein cannot be exported when twin-lysine is present in place of twin-arginine (i.e. a KKR sequence is present).<sup>32</sup> However, studies on the authentic TorA precursor are complicated by the involvement of the assembly chaperone TorD, which plays an important role in export. This may explain why Buchanan *et al.* found the second arginine of the RR-motif to be critical for TorA export, while we observe that an identical RK mutation has little effect on the export of TorA-GFP.

The most important points to emerge from this study are firstly, that the crucial “additional deter-

minants" are located both before and after the RR-motif; and secondly, that their significance can vary, depending on the signal peptide under consideration. This is the case with both the Gram-positive and Gram-negative systems under study here. First, we show for the first time that the -1 serine can be a key determinant in its own right. A relatively conservative substitution of the -1 serine (by alanine) severely inhibits the export of TorA-GFP and DmsA-GFP by both the TatAdCd and TatABC systems. Yet an identical mutation in the SufI signal peptide did not affect export, although a serine>cysteine mutation did.

The results of other studies have already pointed to the presence of important targeting information immediately after the RR-motif, and altering the overall hydrophobicity can affect the export efficiency of Tat substrates *in vivo*.<sup>33</sup> Our studies show that a single hydrophobic residue can have a key role in bacterial Tat signals. In thylakoid Tat signals, it is essential that either the +2 or +3 residue is a hydrophobic residue such as leucine, methionine or valine.<sup>27</sup> In bacteria, phenylalanine is very strongly preferred at the +2 position. The Phe side-chain is not essential for export of SufI, as a +2 leucine supports efficient export of this precursor, whereas a +2 alanine does not.<sup>5</sup> This previous work therefore suggested that hydrophobic residues are strongly preferred at the +2, and perhaps +3 position. Again, our data strongly indicate that the importance of this determinant can vary between different signal peptides. Substitution of the +2 phenylalanine by alanine in the TorA signal peptide has no detectable effect (F14A mutant in Figure 2) and even serine is perfectly acceptable at this position (F14S export efficiency is equally high). There are, however, some constraints regarding the side-chains that are permitted at this position; the F14D mutant is not exported and the F14R mutant is exported at very low levels.

Previous studies on the *S. lividans* TatABC system also pointed to an important role for the +2 residue; substitution of the conserved Phe by a variety of residues resulted in xylanase export efficiencies that varied from 20–40% of the wild-type levels (these effects are given in Table 1, where we have attempted to summarise our data and illustrate previously characterised effects of a variety of Tat signal peptide mutations in several other bacteria and precursor proteins).

Our data obtained with the DmsA signal peptide provide the clearest evidence yet that a hydrophobic residue can be an absolute requirement at this position. This signal peptide has the sequence SRRGLV, and we have found that substitution of the +2 leucine by alanine leads to a complete block in export. Perhaps surprisingly, the subsequent (+3) valine residue is clearly unable to support any level of transport, despite being equally hydrophobic on most hydrophobicity scales. Again, we emphasise the variability of signal peptides; a +2 phenylalanine to alanine substitution in the TorA signal has no detectable effect, implying that a hydrophobic deter-

minant is either not required or provided by the +3 leucine in this signal (SRRRFL).

We conclude that Tat signal peptides contain at least three determinants that, individually, can assume huge importance for the translocation process: a hydrophilic -1 residue (typically serine, threonine, aspartate or asparagine), a conserved twin-arginine motif, in which at least one arginine is a requirement, and a subsequent hydrophobic determinant centred around the +2 residue and perhaps the +3 residue. These determinants appear equally important for export by the *E. coli* TatABC and *B. subtilis* TatAdCd systems.

## Materials and Methods

### Bacterial strains, plasmids and growth conditions

All strains and plasmids used are given in Table 2. *E. coli* MC4100 was used as the parental strain and  $\Delta tatABCDE$ <sup>8</sup> has been described.<sup>34</sup> Arabinose-resistant derivatives were used as described.<sup>11</sup> *E. coli* was grown aerobically in Luria broth at 37 °C. Media were supplemented with (final concentrations) 0.5% (v/v) glycerol, 0.5% trimethylamine *N*-oxide (TMAO), and 1  $\mu$ M ammonium molybdate. Media were supplemented with ampicillin to a final concentration of 100  $\mu$ g/ml and arabinose to 0.5 mM when required.

### DNA techniques

All cloning techniques and transformation of *E. coli* were performed as described.<sup>35</sup> All enzymes used were from Roche Molecular Biochemicals, or Fermentas Life Sciences. The polymerase chain reaction (PCR) was performed using Expand DNA polymerase (Roche). For arabinose-inducible overproduction of *B. subtilis* TatAdCd, plasmid pBADcds was constructed by ligation of the *tatAdCd* operon into the pBAD24 vector previously used to express *E. coli* *tatABC*.<sup>11</sup> The cloning was done in such a way that a C-terminal *Strep*-tag II (underlined) would be attached directly to *tatCd* during the PCR amplification.<sup>36</sup> pBADcds was constructed by ligating an Esp3I/XbaI-cleaved PCR-amplified fragment of *tatAdCd-strep* into NcoI/XbaI-cleaved pBAD24 (J.P.B. *et al.*, unpublished

**Table 2.** Plasmids and strains used in this work

Plasmids	Relevant properties	Reference/ source
pBAD-ABC	pBAD24 derivative containing the <i>E. coli</i> <i>tatABC</i> operon; Amp <sup>r</sup>	11
pBAD-DmsA-GFP	pBAD24 derivative containing DmsA-GFP Amp <sup>r</sup>	11
pEXT-AdCd	pEXT22 derivative containing the <i>B. subtilis</i> <i>tatAdCd</i> operon; Kan <sup>r</sup>	This study
<i>Strains</i>		
<i>E. coli</i>		
MC4100	F <sup>-</sup> $\Delta lacU169$ <i>araD139</i> <i>rpsL150</i> <i>relA1</i> <i>ptsF</i> <i>rbs</i> <i>flbB5301</i>	33
MC4100 $\Delta tatABCDE$	<i>tat</i> deletion strain	8
<i>B. subtilis</i> 168	<i>trpC2</i>	37



**Table 3.** Primers used for mutagenesis of the TorA and DmsA signal peptides

Signal peptide	Primer	
<i>WT-TorA-SRRRFLA</i>		
TorA-SRRRSLA	F-TorAF14S	CATCACGTCGGCGTTCTCTGGCACAACCTC
	R-TorAF14S	GAGTTGTGCCAGAGAACGCCGACGTGATG
TorA-SRRRRLA	F-TorAF14R	CATCACGTCGGCGTCTCTGGCACAACCTC
	R-TorAF14R	GAGTTGTGCCAGACGACGCCGACGTGATG
TorA-SRRRDLA	F-TorAF14D	CATCACGTCGGCGTGATCTGGCACAACCTC
	R-TorAF14D	GAGTTGTGCCAGATCAGGCCGACGTGATG
TorA-SRRRALA	F-TorAF14A	CATCACGTCGGCGTGCTCTGGCACAACCTC
	R-TorAF14A	GAGTTGTGCCAGAGCAGGCCGACGTGATG
TorA-ARRRFLA	F-TorAS10A	CTCTTTCAGGCAGCAGCTCGGCGTTTTC
	R-TorAS10A	GAAAACGCCGACGTGCTGCCTGAAAGAG
TorA-SRRRFAA	F-TorAL15A	CGTCGGCGTTTTGCGGCACAACCTCGGC
	R-TorAL15A	GCCGAGTTGTGCCGAAAACGCCGACG
<i>WT-DmsA-SRRGLV</i>		
DmsA-SRRGFV	F-DmsAAL19F	GTGAGTCGCCGTGGTTTTGTAAAAACGACAG
	R-TorAL19F	CTGTCTGTTTTTACAAAACCGGCGACTCAC
DmsA-SRRGDV	F-TorAL19D	GTGAGTCGCCGTGGTGACGTAAAAACGACAGCGATC
	R-TorAL19D	GATCGCTGTCGTTTTTACGTCACCACGGCGACTCAC
DmsA-SRRGAV	F-TorAL19A	GAGTCGCCGTGGTGCGGTAAAAACGACAG
	R-TorAL19A	CTGTCTGTTTTTACCGCACCGGCGACTC
DmsA-ARRGLV	F-TorAS15A	GGCTGCTGAGGTGGCTCGCCGTGGTTTGG
	R-TorAS15A	CCAAACCGGCGAGCCACCTCAGCAGCC

results). For studies involving the expression of TorA-GFP mutants, the *tatAdCd* operon was expressed using the pEXT22 vector and TorA-GFP was expressed using the arabinose-inducible pBAD24 system (J.P.B. *et al.*, unpublished results). Expression of DmsA-YFP was carried out in a similar manner.

### Mutagenesis of TorA-GFP and DmsA-YFP

The pJDT1 plasmid encoding TorA-GFP was used as a template for QuikChange® Site-directed Mutagenesis.<sup>23</sup> To construct pBAD-DmsA-YFP, the following substitutions were made to pBAD-DmsA-GFP<sup>37</sup> using Quik-change site-directed mutagenesis (Qiagen): V118L, D222G, T252Y, and H280L. Primers used for Quik-Change® site-directed mutagenesis of TorA-GFP and DmsA-YFP (see Table 3).

### SDS-PAGE and Western blotting

*E. coli* cells were fractionated as described,<sup>11</sup> and proteins were separated by SDS-PAGE and immunoblotted using specific antibodies to TatAd (kindly provided by J. Müller) and goat anti-rabbit IgG horse radish peroxidase (HRP) conjugate. The *Strep*-tag II on TatCd was detected directly using a streptactin-HRP conjugate (Institut für Bioanalytik). Sufl, a Tat-dependent substrate of *E. coli*, was visualised using specific antibodies (kindly provided by T. Palmer). An ECL detection kit (Amersham Pharmacia Biotech) was used to visualise the proteins.

### Microscopy

Images were recorded with a Leica DMRE microscope equipped with a Leica TCS SP2 confocal unit and an argon laser. The 488nm laser line was selected and images were recorded with a photomultiplier using the Leica confocal software. Samples were visualised with a 63× magnification oil-immersion objective (numerical aperture 1.4;

Leica). The image size is 512×512 pixels and images were averaged from four successive scans.

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